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Evaluation of the Use of Methylation as a New Tool for the Diagnostics and Progression of Squamous Intraepithelial Lesions

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Abstract: Vaccination against HPV significantly reduced the incidence of HPV-related lesions worldwide. Considering the increasingly younger age of patients in the gynaecological office and the resulting earlier sexual initiation and potentially contact with the HPV virus, doctors need to have tools to verify the diagnosis. Nowadays, women plan motherhood later, so it is necessary to consider whether sexual treatment in the form of, among others, LEEP-conization may increase the risk of premature birth or difficulty dilating the cervix during labour.For this reason, to avoid overtreatment of LSIL changes, methylation testing may be considered. In patients with histopathologically confirmed HSIL during biopsy and ultimately a lower diagnosis - i.e., LSIL or no signs of atypia - methylation turned out to be a useful tool. We performed Pap-smear, HPV genotyping, punch biopsy, LEEP-conization (if needed) and methylation test in 108 women admitted to the District Public Hospital in Poland. Women with a negative methylation test result were significantly more likely to be ultimately diagnosed with LSIL ($p=0.013$). This means that in 85.7% of patients with HSIL, major cervical surgery could have been avoided if methylation was negative. Methylation testing, as well as dual-staining and diagnostics detecting mRNA transcripts of highly oncogenic types of HPV, might be used in future in the diagnosis of pre-cancerous conditions, mainly of the cervix, and in HPV-dependent cervical cancer screening. The methylation test may also be used in the diagnosis and identification of lesions within the cervical canal, including those located deep within the frontal crypts, not visible even during a professional colposcopic evaluation of the cervix, as well as within the crypts of the palatine tonsils - in diagnostics of head and neck diseases.

Keywords: methylation; risk of progression; observation of LSIL and HSIL; fertility-sparing of HSIL; new marker of progression

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1. Introduction

According to the newest worldwide data from GLOBOCAN 2022, cervical cancer has 661,021 new cases per year and more than half of the number of patients die per year (348,189) [1]. Among women's cancers, after breast, lung and colon cancer, it is the most common cancer and the one with the greatest lethality. Which is still surprising in the 21st century and the era of modern diagnostics and prevention. It is the only cancer against which primary prevention is available in the form of vaccination. The introduction of vaccines against HPV has revolutionized cervical cancer prevention [2]. Vaccinations against HPV are included in the primary prevention of precancerous lesions—mainly squamous intraepithelial lesions (SIL) and cervical cancer. The other cancers associated with HPV infections affect the genital organs (vulva, vagina, and penis), anal canal, oral cavity, and upper respiratory tract [3-4]. Vaccination against HPV significantly reduced the incidence of HPV-related

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lesions in New Zealand and the United States. Currently, there are three HPV prophylactic vaccines available commercially in Europe: Gardasil®4 (quadrivalent vaccine against HPV 16, 18, 6 and 11, available since 2006), Cervarix™ (bivalent vaccine against HPV 16 and 18, approved by EMA in 2007 and the FDA in 2009) and Gardasil®9 (nonavalent vaccine against HPV 6, 11, 16, 18, 31, 33, 45, 52 and 58, available since 2014) [5]. Data from clinical trials proved their safety and good preventive effect in people infected with HPV. Nowadays, in Poland, there are two vaccines – bivalent and nonavalent.

As well as the increasing awareness of society contributes to the fact that an increasing percentage of women visiting gynaecological offices are already vaccinated - either in childhood or adulthood. This is related to the growing number of scientific reports supporting the benefits of vaccination in people after sexual initiation and during treatment of SIL [6]. This determines differences in the distribution of detected HPV genotypes in cervical smears and necessitates new cervical triage/screening methods.

The vast majority of cervical cancers are hrHPV-related, and the implication of this virus in cervical cancer pathobiology is well known, namely its effect on the transformation of epithelial surfaces like the squamous–columnar junction of the cervix [7]. Neoplastic transformation starts with HPV DNA integrating into the genome of a normal epithelial cell.

Changing behaviour, earlier initiation, some patients vaccinated - this causes the profile of women with cervical intraepithelial lesions to change. With lower age of patients, doctors' approach to the therapeutic process may change - from less invasive to more conservative. This is related to later decisions about motherhood and the potential impact of the cervical excision procedure, including LEEP-conization, on difficulties in maintaining pregnancy due to cervical scarring. Moreover, some patients co-decide in the therapeutic process and want more conservative treatment - for these patients, they seek methods to monitor early changes in intraepithelial neoplasia, such as LSIL or HSIL, are important.

Such methods include methylation or CINtec testing based on dual-staining or mRNA transcripts detection. The CINtec PLUS test is an immuno-cytochemical test that examines the expression of p16 protein and Ki-67 protein in cervical epithelial cells. The level of p16 protein increases in cells infected with the HPV virus, which is the main factor of cervical cancer. In turn, the amount of Ki 67 protein increases in rapidly dividing cells, such as cancer cells. Testing both markers simultaneously increases the sensitivity of the test and allows for the differentiation of changes occurring in cervical cells, detecting cancerous changes caused by the highly oncogenic human papillomavirus (HPV) at an early stage of cancer development. DNA methylation, one of the most extensively studied epigenetic mechanisms regulating gene expression, has emerged as a promising source for noninvasive disease biomarkers. In the context of cervical cancer, alterations in promoter methylation levels of various genes—both human and HPV-related—have been linked to HPV status, lesion progression, and patient outcomes [8-10].

There is evidence, that type-specific DNA methylation shows good risk stratification for HPV-positive women and improved performance compared with HPV16/18/31/45 genotyping and Pap-smear, suggesting that HPV DNA methylation has potential for clinical use as a triage test for HPV-positive women. Our study aims to demonstrate that the methylation test applies to patients with histopathological confirmed squamous intraepithelial neoplasia of the cervix.

2. Materials and Methods

2.1. Study Design

We provide a prospective, ongoing 24-month, non-randomised study in patients reporting to the District Public Hospital in Poznan, Poland and Individual Specialized Gynecological Practice for an in-depth diagnostic of squamous intraepithelial lesions. The study group included patients referred for in-depth diagnostics, i.e. colposcopy with cervical biopsy and curettage of the cervical canal, who were qualified based on meeting at least one of the following criteria: 1) abnormal cytology result, 2) positive test result for the highly oncogenic HPV virus (especially 16, 31 and 18), 3) clinically abnormal image of the cervix. Abnormal LBC result means ASC-US (atypical squamous cells of undetermined significance), AGC (atypical glandular cells), LSIL (low-grade squamous intraepithelial lesions), HSIL (high-grade squamous intraepithelial lesions), cervical cancer suspicion. In the entire group of patients, a cytodiagnostic test was performed, i.e. a cervical smear indicating the presence of methylation. Standardly, LEEP-conization was performed in cases of histopathologically confirmed HSIL in cervical biopsy. In some cases, e.g. clinical suspicion of a highly advanced lesion, the cervical biopsy stage was omitted and LEEP-conization was performed. In some clinical situations, a hysterectomy was performed instead of LEEP-conization of the cervix. For the final histopathological diagnosis, the highest diagnosis from biopsy, LEEP-conization or hysterectomy was considered. Patients whose histopathological final results was negative for squamous intraepithelial lesions were included in the control group.

2.2. Specimen Collection and Handling

2.2.1. HPV Genotyping Test and LBC

The BD Onclarity HPV Assay is a method for detecting 14 different HPV genotypes while incorporating a β-globin internal control (IC) as a processing control. The HPV genotypes are detected using specific primers designed to target a region of 79 to 137 bases in the E6/E7 genome, while the IC primers am. In contrast, a 75-base region in the human β-globin gene. To perform the assay, three polymerase chain reaction (PCR) assay tubes, namely G1, G2, and G3, are utilized, along with four optical channels for the detection process. The HPV genotypes that can be detected individually are HPV 16, 18, 31, 45, 51, and 52. The remaining genotypes are grouped as follows: P1 - HPV 33/58, P2 - 56/59/66, and P3 - 35/39/68. The IC serves as a control to ensure the accuracy and validity of the assay.

2.2.2. Sample Preparation for Methylation Test

A. Isolation from cytological cervical material

Genomic DNA was extracted from the Cell Collection Medium 20 ml (Roche Diagnostics) using TANBead OptiPure Viral DNA/RNA Kit based on magnetic bead technology. The extraction was carried out on TANBead Maelstrom™ 4800 Nucleic Acid Extraction System from Taiwan Advanced Nanotech. Sample preparation for the extraction stage: 3 ml of the sample from the Cell Collection Medium was centrifuged for 5 minutes at 7000 r.p.m to obtain a cell pellet. The supernatant was removed to avoid damaging the pellet at the bottom of the test tube. Then the sediment was washed with Tris buffer (SIGMA TRIS SALINE) and centrifugated for 5 minutes at 7000 r.p.m. The supernatant was removed, and the pellet was suspended with the 300 μl or 400 μl lysis buffer (GeneMAP Extraction Buffer), depending on the number of pellets, and 10 μl Proteinase K. Next the sample was incubated for 90 minutes at a temperature of 56°C. After incubation, the material was used directly for the isolation step on TANBead Maelstrom™ 4800 Nucleic Acid Extraction System. DNA concentration of each sample was measured using a NanoReady Touch Series Micro Volume (UV-Vis) Spectrophotometer from Lifereal Biotechnology Co, Ltd.

B. DNA bisulfite-conversion and methylation

DNA samples were subjected to DNA denaturation and bisulfite conversion using a Sample Pretreatment Kit for DNA Methylation Test. The methylation step was performed by DNA Methylation Detection Kit for Human PAX1, SOX1, and HAS1 Gene (Real-time PCR) Yaneng BIOscience (Shenzhen) Co., Ltd. The Sample Pretreatment Kit for the DNA Methylation test procedure comprises the following steps:

1. Denaturation and Bisulfite Conversion,
2. Binding of the DNA to the spin column and Desulfonation,
3. Washing the spin column bound DNA and elimination of ethanol,
4. Elution of converted DNA.

The methylation kit is based on multiplex real-time methylation-specific assay for the detection of promoter hypermethylation of the genes PAX1, SOX1, and HAS1, in human cervical specimens.

After bisulfite treatment, the primes and probes can distinguish between methylated and non-methylated sequences. The Tagman probes can correspond to the sequences that detect methylation in bisulfite-treatment DNA samples. ACTB was used as a reference gene for bisulfite treatment and DNA input. For each run positive control and negative control for the operation process and environmental pollution monitoring. The limit of detection (LOD) of the DNA Methylation Detection Kit for Human PAX1, SOX1, and HAS1 is 1% for each gene. Real-time PCR was performed using a Gentier96R Real-time PCR System (Xian Tianlong Science and Technology Co., Ltd). The analysis results were conducted according to the manufacturer's manual.

2.3. Colposcopy, Punch Biopsy and LEEP-Conisation

Further validation of abnormal screening results was performed on all patients with an abnormal smear above the ASCUS (as follows: ASC- US, AGC, LSIL, HSIL, cervical cancer), a positive HPV test for types 16, 18, 31, and a clinically suspicious cervical image. The Polish Society of Colposcopy and Cervical Pathophysiology recommended the International Federation of Cervical Pathology and Colposcopy classification. The colposcopic assessment was performed by two independent colposcopists, including one - a gynecological oncologist with 15 years of experience in optical stereoscopic colposcope.

2.4. Statistical Analysis

Analysis was conducted with statistical software R, version R4.1.2. All analyses assumed a significance level of $\alpha = 0.05$. Nominal variables were presented as n and %, age was presented as median with quartiles 1 and 3 due to non-normal distribution. The normality of distribution was analysed with Shapiro-Wilk's test and further verified with skewness and kurtosis. Comparisons were made with the Mann-Whitney U test, Pearson's chi-square test and Fisher exact test, as appropriate.

3. Results

The analysis included 108 women with an average age of 40 years. Among the group, 85 women belonged to the study group and 23 to the control group. The control group consisted of patients whose histopathological diagnosis showed no pathology. Table 1 shows the characteristics of the patients divided into the study and control groups. The methylation outcome (positive/negative) was significantly different between the groups, p = 0.004. The research group was characterized by a higher proportion of positive outcomes (43.5% vs 8.7%, n = 2) and a lower proportion of negative outcomes (56.5% vs 91.3%).

HPV outcome (positive/negative) was significantly different between the groups, p < 0.001. The research group was characterized by a higher proportion of positive HPV outcomes (85.9% vs 34.8%, n = 8) and a lower proportion of negative HPV outcomes (14.1% vs 65.2%). Analysis of HPV genotypes confirmed that incidences of genotype 16 and genotype 31 differed significantly between the groups, p = 0.011 and p = 0.038, respectively.

Table 1. Study groups characteristics and comparison of groups.

Characteristics	Research Group	Control Group	MD (95% CI)	p
N	85 (100.0)	23 (100.0)	-	-
Age, years, Me (Q1; Q3)	37.97 (33.79;41.92)	49.47 (41.87;58.52)	-11.50 (-16.39;-5.82)	**< 0.001**
Methylation, n (%)				
Positive	37 (43.5)	2 (8.7)	-	**0.004**

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Characteristics	Research Group	Control Group	MD (95% CI)	p
Negative	48 (56.5)	21 (91.3)		
Pap-smear, n (%)				
NILM	9 (10.6)	13 (56.5)	-	**< 0.001**
ASC-US	15 (17.6)	4 (17.4)		
LSIL	21 (24.7)	3 (13.0)		
ASC-H	22 (25.9)	1 (4.3)		
HSIL	14 (16.5)	0 (0.0)		
AGC	2 (2.4)	1 (4.3)		
Clinically suspicious image of the cervix	2 (2.4)	1 (4.3)		
HPV, n (%)				
Positive	73 (85.9)	8 (34.8)	-	**< 0.001**
Negative	12 (14.1)	15 (65.2)		
HPV genotype, n (%)*				
HR	34 (40.0)	6 (26.1)	-	0.326
16	38 (44.7)	3 (13.0)	-	**0.011**
18	4 (4.7)	0 (0.0)	-	0.576
31	14 (16.5)	0 (0.0)	-	**0.038**
45	5 (5.9)	0 (0.0)	-	0.582
Biopsy, n (%)				
LSIL	16 (18.8)	0 (0.0)	-	**< 0.001**
HSIL	58 (68.2)	0 (0.0)		
Adenocarcinoma	2 (2.4)	0 (0.0)		
Squamous cell carcinoma	2 (2.4)	0 (0.0)		
No signs of atypia	3 (3.5)	13 (56.5)		
Not performed	4 (4.7)	10 (43.5)		
LEEP/hysterectomy, n (%)				
LSIL	15 (17.6)	0 (0.0)	-	**< 0.001**
HSIL	56 (65.9)	0 (0.0)		
Adenocarcinoma	2 (2.4)	0 (0.0)		
Squamous cell carcinoma	2 (2.4)	0 (0.0)		
No signs of atypia	7 (8.2)	5 (21.7)		
Not performed	3 (3.5)	18 (78.3)		
Final, n (%)				

Characteristics	Research Group	Control Group	MD (95% CI)	p
LSIL	15 (17.6)	0 (0.0)		
HSIL	66 (77.6)	0 (0.0)		
Adenocarcinoma	2 (2.4)	0 (0.0)	-	**< 0.001**
Squamous cell carcinoma	2 (2.4)	0 (0.0)		
No signs of atypia	0 (0.0)	23 (100.0)		

Me – median, Q1 – first quartile, Q3 – third quartile, MD – median difference (research group vs control group); N- number. Comparisons were made with the Mann-Whitney U test (age), Pearson's chi-square test (methylation, HPV positive/negative, HPV HR, HPV 16) or Fisher exact test (other characteristics). * Sum exceeded 100% as patients could belong to multiple groups.

3.1. Comparison between Patients with a Positive Outcome of Methylation (+) and Patients with a Negative Outcome of Methylation (-) in the Research Group

Groups differed with pap-smear outcome in a significant way, p = 0.016. ASC-H and HSIL were more common among patients with positive outcomes of methylation (32.4% vs 20.8%, 29.7% vs 6.2%, n = 3, respectively). NILM, ASC-US and LSIL were less commonly observed among patients with positive outcomes of methylation (8.1%, n = 3 vs 12.5%, n = 6, 8.1%, n = 3 vs 25.0% and 16.2%, n = 6 vs 31.2%). HPV genotype 16 was more common among patients with positive outcomes of methylation (59.5% vs 33.3%), p = 0.029.

Groups differed with LEEP/hysterectomy outcome in a significant way, p = 0.019. HSIL was more common among patients with positive outcomes of methylation (75.7% vs 58.3%). LSIL was less commonly observed among patients with positive outcomes of methylation (8.1%, n = 3 vs 25.0%). Adenocarcinoma and clinically suspicious outcomes were only observed among patients with a positive outcome of methylation, both with a proportion of 5.4% (n = 2). Patients with no signs of atypia were less common in the group with positive outcomes of methylation (2.7%, n = 1 vs 12.5%, n = 6).

Groups differed with the outcome in a significant way, p = 0.048. LSIL was less common among patients with positive outcomes of methylation (10.8%, n = 4 vs 22.9%). The proportion of HSIL was similar in both groups (78.4% vs 77.1%). Adenocarcinoma and clinically suspicious images of the cervix were only observed among patients with a positive outcome of methylation, both with a proportion of 5.4% (n = 2), as presented in Table 2.

Table 2. Comparison of patients with positive outcome of methylation (+) and negative outcome of methylation (-) in both study groups.

Characteristics	Research group			Control group		
	Methylation		p	Methylation		p
	+	-		+	-	
PAP, n (%)						
NILM	3 (8.1)	6 (12.5)		1 (50.0)	12 (57.1)	
ASC-US	3 (8.1)	12 (25.0)		0 (0.0)	4 (19.0)	
LSIL	6 (16.2)	15 (31.2)	**0.016**	0 (0.0)	3 (14.3)	0.332
ASC-H	12 (32.4)	10 (20.8)		1 (50.0)	0 (0.0)	
HSIL	11 (29.7)	3 (6.2)		0 (0.0)	0 (0.0)	

Characteristics	Research group			Control group		
	Methylation		p	Methylation		p
	+	-		+	-	
AGC	1 (2.7)	1 (2.1)		0 (0.0)	1 (4.8)	
Clinically suspicious image of the cervix	1 (2.7)	1 (2.1)		0 (0.0)	1 (4.8)	
HPV negative, n (%)	3 (8.1)	9 (18.8)	0.279	1 (50.0)	14 (66.7)	> 0.999
HPV genotype, n (%)*						
16	22 (59.5)	16 (33.3)	**0.029**	0 (0.0)	3 (14.3)	> 0.999
18	2 (5.4)	2 (4.2)	> 0.999	0 (0.0)	0 (0.0)	-
31	5 (13.5)	9 (18.8)	0.726	0 (0.0)	0 (0.0)	-
Biopsy, n (%)						
LSIL	5 (13.5)	11 (22.9)		0 (0.0)	0 (0.0)	
HSIL	25 (67.6)	33 (68.8)		0 (0.0)	0 (0.0)	
Adenocarcinoma	2 (5.4)	0 (0.0)	0.208	0 (0.0)	0 (0.0)	> 0.999
Squamous cell carcinoma	2 (5.4)	0 (0.0)		0 (0.0)	0 (0.0)	
No signs of atypia	2 (5.4)	1 (2.1)		1 (50.0)	12 (57.1)	
Not performed	1 (2.7)	3 (6.2)		1 (50.0)	9 (42.9)	
LEEP/hysterectomy, n (%)						
LSIL	3 (8.1)	12 (25.0)		0 (0.0)	0 (0.0)	
HSIL	28 (75.7)	28 (58.3)		0 (0.0)	0 (0.0)	
Adenocarcinoma	2 (5.4)	0 (0.0)	**0.019**	0 (0.0)	0 (0.0)	0.395
Squamous cell carcinoma	2 (5.4)	0 (0.0)		0 (0.0)	0 (0.0)	
No signs of atypia	1 (2.7)	6 (12.5)		1 (50.0)	4 (19.0)	
Not performed	1 (2.7)	2 (4.2)		1 (50.0)	17 (81.0)	
Final, n (%)						
LSIL	4 (10.8)	11 (22.9)		0 (0.0)	0 (0.0)	
HSIL	29 (78.4)	37 (77.1)		0 (0.0)	0 (0.0)	
Adenocarcinoma	2 (5.4)	0 (0.0)	**0.048**	0 (0.0)	0 (0.0)	-
Squamous cell carcinoma	2 (5.4)	0 (0.0)		0 (0.0)	0 (0.0)	
No signs of atypia	0 (0.0)	0 (0.0)		2 (100.0)	21 (100.0)	

Comparisons were made with Pearson's chi-square test (HPV negative, HPV 16, HPV 31 in research group) or Fisher exact test (other characteristics). * Sum exceeded 100% as patients could belong to multiple groups.

3.2. Effectiveness of Methylation in Predicting Final Outcome in the Research Group

The sensitivity of methylation when predicting the final outcome was 47.14% (CI95%: 35.09%-59.45%), while specificity was 73.33% (CI95%: 44.90%-92.21%), as shown in Table 3.

Table 3. Sensitivity and specificity of methylation in predicting final outcome in research group.

		Final outcome						
		HSIL/adenocarcinoma/squamous cell carcinoma	LSIL	**Sensitivity, %**	**Specificity, %**	**PPV, %**	**NPV, %**	**Accuracy, %**
Methylation	+	33	4	47.14 (35.09-59.45)	73.33 (44.90-92.21)	89.19 (77.47-95.19)	22.92 (16.94-30.24)	51.76 (40.66-62.74)
	-	37	11					

PPV – positive predictive value, NPV – negative predictive value.

3.3. Effectiveness of Methylation in Predicting Final Outcome in both Study Groups (Research Group + Control Group)

The sensitivity of methylation when predicting the final outcome was 47.14% (CI95%: 35.09%-59.45%), while specificity was 84.62% (CI95%: 69.47%-94.14%), as presented in Table 4.

Table 4. Sensitivity and specificity of methylation in predicting final outcome in both study groups. (research group + control group).

		Final outcome						
		HSIL/adenocarcinoma/squamous cell carcinoma	LSIL/no signs of atypia	**Sensitivity, %**	**Specificity, %**	**PPV, %**	**NPV, %**	**Accuracy, %**
Methylation	+	33	6	47.14 (35.09-59.45)	84.62 (69.47-94.14)	84.62 (71.67-92.28)	47.14 (40.78-53.60)	60.55 (50.73-69.78)
	-	37	33					

PPV – positive predictive value, NPV – negative predictive value.

Patients with no biopsy result or no LEEP-conization result were excluded from the following analysis, i.e., only patients with complete histopathological results were included. Among patients with HSIL diagnosed in biopsy and LSIL confirmed during the LEEP-conization procedure, statistically significantly more patients had a negative methylation result than a positive one (p=0.013). The sensitivity of the methylation results in predicting a lower final diagnosis for biopsy result HSIL was 91.67%, as shown in Table 5 and Table 6.

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Table 5. Comparison of patients with HSIL → LSIL/no signs of atypia and other patients.

Characteristics	Biopsy = HSIL & LEEP = LSIL/ no signs of atypia n = 12	Other patients n = 67	p
Methylation, n (%)			
Negative	11 (91.7)	32 (47.8)	**0.013**
Positive	1 (8.3)	35 (52.2)	

Comparisons made with Pearson's chi-square test.

Table 6. Sensitivity and specificity of methylation in predicting biopsy/LEEP outcome.

		Biopsy = HSIL & LEEP = LSIL/no signs of atypia	Other patients	Sensitivity, %	Specificity, %	PPV, %	NPV, %	Accuracy, %
Methylation	-	11	32	91.67 (61.52-99.79)	52.24 (39.67-64.60)	25.58 (20.25-31.76)	97.22 (84.09-99.57)	58.23 (46.59-69.23)
	+	1	35					

PPV – positive predictive value, NPV – negative predictive value.

In the case of patients with a histopathological diagnosis of LSIL in a biopsy, in whom the final diagnosis was confirmed, the methylation result was negative in 85.7% and positive in 14.3%, but no statistically significant results were obtained (p=0.119), as presented in Table 7 and Table 8.

Table 7. Comparison of patients with LSIL → LSIL/no signs of atypia and other patients.

Characteristics	Biopsy = LSIL & LEEP = LSIL/no signs of atypia n = 7	Other patients n = 72	p
Methylation, n (%)			
Negative	6 (85.7)	37 (51.4)	0.119
Positive	1 (14.3)	35 (48.6)	

Comparisons made with Fisher's exact test.

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Table 8. Sensitivity and specificity of methylation in predicting biopsy/LEEP outcome.

		Biopsy = LSIL & LEEP = LSIL/no signs of atypia	Other patients	Sensitivity, %	Specificity, %	PPV, %	NPV, %	Accuracy, %
Methylation	-	6	37	85.71 (42.13-99.64)	48.61 (36.65-60.69)	13.95 (10.01-19.12)	97.22 (84.88-99.54)	51.90 (40.36-63.29)
	+	1	35					

PPV – positive predictive value, NPV – negative predictive value.

4. Discussion

Our study aimed to evaluate the methylation test as a modern predictive tool for use in patients with histopathologically confirmed cervical squamous intraepithelial neoplasia. Considering the increasingly younger age of patients in the gynaecological office and the resulting earlier sexual initiation and potentially contact with the HPV virus, the doctor must have tools to verify the diagnosis. Nowadays, women plan motherhood later, so it is necessary to consider whether sexual treatment in the form of, among others, LEEP-conization may increase the risk of premature birth or difficulty dilating the cervix during labour [11-12].

For this reason, to avoid overtreatment of LSIL changes, methylation testing may be considered. In patients with histopathologically confirmed HSIL during biopsy and ultimately a lower diagnosis - i.e., LSIL or no signs of atypia - methylation turned out to be a useful tool. Women with a negative methylation test result were significantly more likely to be ultimately diagnosed with LSIL (p=0.013). This means that in 85.7% of patients with HSIL, major cervical surgery could have been avoided if methylation was negative. However, no significant results were obtained in the case of LSIL biopsy results. This means that methylation is not yet a tool for monitoring CIN 1 lesions, even though the discrepancy in the results seemed to be large. It would probably be worth repeating this study on a larger group of patients. The high percentage of results consistent with both targeted biopsy and LEEP-conization proves the high quality of colposcopy performed in our center, without overdiagnosis and overtreatment, but also without underestimation.

Salta S. and co-authors published the results of a meta-analysis regarding DNA methylation as a triage marker for colposcopy referral in HPV-based cervical cancer screening [12]. They indicate the need to find an accurate test which might identify clinically relevant hrHPV infections is key to reducing the number of unneeded referrals and interventions (with associated risks and costs) as well as hrHPV test repetitions [14-15]. Considering sensitivity and specificity for CIN2+ detection were 68% (CI 95% 63–72%) and 75% (CI 95% 71–80%), respectively. In our study, sensitivity and specificity of methylation in predicting LSIL outcome in LEEP-conization outcome achieved 91.67% and 52.67% respectively. In a 2024 paper, Hoyer's team of researchers and co-authors made preliminary confirmation possibility of using methylation in detecting the recurrence of HSIL - CIN2 lesions and CIN 3. The sensitivity of the methylation test was 67%, compared to the hrHPV test - 83% and specificity was 90% and 62%, respectively, in favour of the test GynTect methylation and these results were statistically significant. The authors also confirmed the higher diagnostic value of the combined methylation test and cytology test compared to the combined hrHPV molecular test and cytology test. However, it is northworthing a significant limitation of this study, as the size of the study group was only 17 cases [15]. Methylation tests can also be a complementary diagnosis in the case of persistent infection with HPV types 16, 18 and 59. In the study by Peronace et al., the percentage of positive methylation results increased with more abnormal cytological test results, for the diagnosis of HSIL it was over 84% [17]. In 2023, LI et al conducted research on a large group of patients – 476 women. PAX1m was significantly increased in HSIL, especially in cervical cancer, but there was no significant difference between cervical intraepithelial neoplasms CIN 1 and CIN2. However, HPV VL significantly differed between CIN1 and CIN2 but not between CIN3 and cervical cancer [18]. Most publications confirm the need to use methylation based on co-testing or as an additional diagnostic measure in the event of abnormal screening results. In a 2019 study by del Pino et al., the high sensitivity and specificity of the methylation test was confirmed, which was 84.6% and 74%, respectively, in detecting HSIL changes - CIN2 and CIN 3 and cervical cancer. The combined test with molecular diagnostics had even higher sensitivity and specificity of 80.7% and 85.1%, respectively. The methylation rate of CADM1, MAL and miR124 increases with the severity of the lesion [19]. In recent years, DNA methylation-based biomarkers have been explored as potential tools for triaging hrHPV-positive cases, aiming to reduce the number of referrals for colposcopy and prevent overdiagnosis and overtreatment. However, the evidence supporting these triage tests remains limited, as noted in the latest World Health Organization recommendations [20]. To address this, we conducted a study to assess the value of DNA methylation-based biomarker in the Polish population.

5. Conclusions

Methylation testing, as well as dual-staining and diagnostics detecting mRNA transcripts of highly oncogenic types of HPV, might be used in future in the diagnosis of pre-cancerous conditions, mainly of the cervix, and in HPV-dependent cervical cancer screening. Moreover, it may become a tool based on molecular and genetic tests, perhaps based on risk algorithms for the development and progression of SIL and cervical cancer. Detailed analysis of the surface topography of squamous intraepithelial lesions in the colposcopic examination with the support of AI and in combination with the results of methylation tests could reduce the number of LEEP-conization procedures in the population of young patients without offspring. Groups of experts and reviewers should create new guidelines for conduct in the era of widespread and available vaccination against HPV in the vaccinated population. The methylation test may also be used in the diagnosis and identification of lesions within the cervical canal, including those located deep within the frontal crypts, not visible even during a professional colposcopic evaluation of the cervix, as well as within the crypts of the palatine tonsils - in diagnostics of head and neck diseases. The authors believe that confirmation of the use of methylation in cervical diagnostics will begin the work of scientists in searching for new types of methylation detecting atypical and cancerous changes in the glandular epithelium.

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